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Alteration of the bacterial peptidoglycan layer to overcome vancomycin-resistance

Alison Yirinec

Introduction:

Human bodies naturally contain trillions of bacteria, many of which are harmless and aid in everyday functioning. Bacterial cells outnumber human cells ten to one in a single person's body. At a given time, there are more than five hundred different bacterial species living inside a person that work towards keeping the immune system healthy and extracting energy and nutrients from food.¹ Though bacteria have useful applications in human bodies, they can cause serious illness provided the correct set of risk factors. For example, *Enterococci* bacteria are naturally found in healthy human digestive systems. Sometimes these seemingly harmless bacteria can result in fatal infections when a person possesses certain risk factors, like having a compromised immune system or recovering from surgery.² Additionally, there are many bacteria that are naturally infectious. These bacteria replicate quickly upon entering the body and cause damage to the tissue through the release of chemicals called toxins.²

Growth of bacteria occurs at the thick peptidoglycan layer, which is an important component of the bacterial cell wall.³ With its strength and flexibility, the peptidoglycan layer aids in the maintenance of the cell's shape and the attachment of cell components to the cell wall and plasma membrane. It is composed of a sugar backbone and pentapeptide side chains.⁴ In the biosynthetic pathway to synthesize the peptidoglycan for many bacteria, L-alanine undergoes a chiral conversion into D-alanine by the enzyme D-alanine racemase.⁵ Next, in an ATP-dependent step, two

D-alanine molecules bind together by a D-alanine:D-alanine ligase to form a dipeptide. The coupled product is then attached to the first three amino acids of the side chain through the enzymatic activity of ligase MurF. Once formed, the pentapeptide chain is latched onto a lipid, Lipid II, and is flipped out of the cytoplasm to the outer membrane. There, it is added to the growing peptidoglycan layer through transglycosylation, with the D-Alanine-D-Alanine forming the C-terminus of the pentapeptide. Once incorporated into the cell wall, cleavage of the fifth position amino acid by the transpeptidase allows for cross-linking.⁴

When infections do occur from bacteria, the typical treatment is antibiotics. Antibiotics work by either killing the bacteria or inhibiting its growth, with different classes of antibiotics functioning by distinctive methods. This is done by taking advantage of the main variations that exist between human cells and bacterial cells: bacteria have cell walls while human cells do not and the mechanisms to make proteins and copy DNA deviate between the two. Some prevent growth by interfering with the peptidoglycan layer mechanism that builds cell walls, while others kill the bacteria by either preventing bacterial ribosomes from building proteins or by irreversibly destroying the DNA.⁶ When the bacterial cell no longer has the ability to multiply or is permanently damaged, the body's natural immune system will be better able to fight the infection.⁷

Antibiotics may be strong weapons against infectious diseases, but infections still remain the second-leading cause of death in the world. In recent years, the world has been moving towards the threat of multidrug-resistant microbes.⁸ Since its discovery, antibiotics have been misused and overused allowing bacteria to

develop a resistance. In recent years, the rate of resistance development has increased.⁸ Bacteria resistance arises mainly from two unique mechanisms. For some bacteria, alterations in the actual structure of the binding site of the bacterial peptidoglycan layer prevent the antibiotic-bacteria complex from forming, while other modified bacteria function by directly neutralizing the antibiotic.⁹

The rate of development of these modifications is due to the fast rate that bacteria are able to adapt. The rate of bacterial adaptation far exceeds that of humans, which is attributed to their increased rate of replication. Bacteria have the ability to reproduce in approximately half an hour, compared to the twenty to thirty years that most humans take to reproduce.⁸ If one modified bacteria cell is present and survives an antibiotic treatment, it will reproduce rapidly forming a population of resistant bacteria. Acquiring the initial resistance is done by genetic mutations to the bacteria or by obtaining DNA pieces that code resistance.⁹ These alterations become significant when the altered bacteria undergo rapid replication, causing bacterial infections to become an increasing danger. Infections spread quickly and are becoming increasingly more difficult to treat.⁹

Glycopeptide antibiotics are a group of antibiotics that consist of both a carbohydrate portion and amino acid residues.¹⁰ They tend to be large and rigid, and they act as inhibitors to the crosslinking of the bacterial peptidoglycan layer. Inhibition occurs when the peptide section of the antibiotic binds to the D-Ala-D-Ala dipeptide at the terminal end of the peptidoglycan pentapeptide side chain. This binding site is highly specific to the D-Ala-D-Ala dipeptide portion after it has attached to the Lipid II and has flipped to the outer membrane. As this dipeptide is

most commonly found in bacterial cell walls, the toxicity of glycopeptide antibiotics is very selective.¹⁰ At the binding site, a complex is formed with hydrogen bonds between the peptide section of the antibiotic and the dipeptide. When bonded, steric interference arises from the bulkiness of the glycopeptide structure. As a result, the formation of the peptidoglycan layer's sugar backbone and the cross-linking process to strengthen the cell wall are both hindered, halting the mechanisms of the biosynthetic pathway.¹⁰ Without the ability for the peptidoglycan layer to grow and strengthen, the bacteria itself will die.

Vancomycin was the first glycopeptide antibiotic created and is currently the main treatment option for Gram-positive bacteria, which has a thicker peptidoglycan layer than Gram-negative bacteria.¹⁰ It is unable to enter the cytoplasm of the bacteria, and therefore interferes with the biosynthetic process at the transglycosylation step, which involves the addition of the peptidoglycan precursor onto the growing peptidoglycan layer. Vancomycin attaches directly to the dipeptide of the peptidoglycan layer, rather than the enzymes involved with the cell wall synthesis. As a result, vancomycin's ability to fight bacterial infections is determined by the enzymes that control the terminal dipeptide of the pentapeptide chain. If the bacterium has an enzyme with high substrate specificity to D-alanine, then vancomycin has a strong ability to inhibit the bacterial growth.¹¹

There are two processes in bacteria that, in conjunction, result in resistance to the antibiotic vancomycin: modification of the target C-terminus dipeptide and the removal of the D-Ala-D-Ala binding site completely. To modify the terminus, the D-alanine in the fifth position is replaced by either D-lactate or D-serine.

Glycopeptides have a much lower affinity to the altered dipeptides, with the ability to bind decreased by three orders of magnitude.^{2,11} D-serine as the terminal amino acid has a high degree of steric interference, preventing vancomycin from being able to bind. D-lactate results in the loss of a hydrogen bond, destabilizing the complex.¹¹

There are three acquired resistant mechanisms that replace the dipeptide with D-alanine-D-lactate. They are called VanA, VanB, and VanD. For VanA, the most common type, the D-lactate replaces the D-alanine when the enzyme VanH forms D-lactate through the reduction of pyruvate. The VanA ligase subsequently acts as a catalyst to form an ester bond between the D-alanine and the newly formed D-lactate, resulting in an altered dipeptide that is now capable of binding to the peptidoglycan layer.¹¹ Two acquired resistant mechanisms that have D-serine as the amino acid replacing the D-alanine are VanE and VanG.¹¹ In addition to forming the new dipeptide, the bacteria must also remove the original binding site of vancomycin. The enzymes VanX and VanY work together to accomplish this, with VanX hydrolyzing the D-Ala-D-Ala peptide bond and VanY eliminating the D-alanine from the fifth position of the peptidoglycan layer. VanX specifically functions as an intracellular enzyme.¹¹

VanC resistance, which is intrinsic rather than acquired, can also cause D-serine or D-lactate at the peptidoglycan terminus. The three genes involved are *vanT*, *vanC*, and *vanXYc*. A racemase enzyme, which is required for the production of the amino acid, is encoded from the *vanT* gene. The racemase enzyme does a chiral conversion of the L amino acid to the D amino acid. A peptide bond is then formed between D-alanine and the unnatural amino acid by ligase VanC. VanXYc hydrolyzes

the D-Ala-D-Ala bond, producing free D-alanine to bind with the new, unnatural amino acid and ensuring D-Ala-D-Ala is not the primary precursor formed.¹¹

Two bacteria that are innately resistant to vancomycin are *Lactobacillus casei* and *Lactobacillus plantarum*. These strains are Gram-positive probiotics that are naturally found in the human body. Probiotics provide health benefits, including their ability to aid in immunity. Typically, the intestines provide the optimal environment for lactobacilli to grow.¹² Lactobacilli are intrinsically resistant to vancomycin and other glycopeptides, as the precursor D-lactate is utilized in the formation of the pentapeptide. As a result, D-Ala-D-Lac is located at the C-terminus. In *L. plantarum*, an additional enzyme, D-Ala-D-Ala-dipeptidase, will actively degrade the dipeptide D-Ala-D-Ala.¹³ This process works in conjunction with the enzyme VanXYc to ensure that the dipeptide incorporated into the peptidoglycan layer is D-Ala-D-Lac.¹¹ It also contains transpeptidase, which in the presence of unnatural amino acids will cleave the pentapeptide between the fourth and fifth positions after it has been attached to the peptidoglycan layer. The new amino acid will then be incorporated into the peptidoglycan layer.⁴

By finding a method to alter the peptidoglycan layer and reform the initial D-Ala-D-Ala C-terminus, vancomycin resistance can be overcome. To develop such a technique, the bacterium *L. casei* was utilized. Using viability assays, it was determined if the D-lactate in the fifth position was capable of being replaced by D-alanine. If the D-alanine could out compete the D-lactate in the formation of the dipeptide, then the necessary D-Ala-D-Ala binding site would be present for vancomycin to bind to and growth would be inhibited. For use in the body, 5mM of

D-alanine is the highest concentration obtainable in the blood, though 1mM is preferable, and up to 20mM D-alanine is feasible for topical wounds.¹⁴

Experimental Methods:

Initially, the resistance of the *L. casei* was tested by supplementing the bacteria with 256mg/L vancomycin only in 1mL of MRS broth. The solution was then incubated in the shaker overnight and, after 24 hours, analyzed for the level of growth. The same procedure was performed for incubating the bacteria with D-alanine only, at concentrations of 100mM, 20mM, 18mM, 16mM, 14mM, 12mM, 10mM, and 5mM.

A series of viability assays were then performed in 96-well plates, with incubation overnight in the shaker to allow the bacteria time to grow. Optical densities were determined after 24 hours of growth time. For each assay, every well contained a total of 200 μ L. The assays were run at varying concentrations of vancomycin and D-alanine, with the concentration of one component kept constant while the concentration of the other was varied over a range.

For the bacteria cells, vancomycin, and D-alanine, necessary dilutions were prepared prior to plating. The solutions for vancomycin were made at two times the desired concentration and 100 μ L was pipetted into each well. The bacterial cells and the D-alanine were made at four times the desired concentration and 50 μ L of each was pipetted into each well. This was to account for the dilution that occurred when all the components were combined. The vancomycin stock solutions were 10mg/mL. To prepare them, 5mg of vancomycin was weighed in a 1.5mL Eppendorf PCR tube. Then it was dissolved in 0.5mL of distilled water. The solution was

buffered with 1-2 drops of 10M NaOH_(aq) and stored in the freezer. For each assay, the vancomycin stock was diluted in MRS broth to the desired concentration. The D-alanine solutions were prepared fresh each day by making a stock and serially diluting it. For the bacteria cells, an overnight growth was prepared in 5mL of MRS broth. It was diluted to 1:20 by pipetting 500µL into 9.5mL of MRS broth, shaken, and immediately plated. Multichannel pipettes were used for the plating of the bacteria to prevent it from settling. This ensured that all wells received an equivalent concentration of starting bacteria and therefore lowered the possibility of error. All assays were performed in triplicates.

Assays were also performed for *Bacillus subtilis*, a non-vancomycin resistant bacterium, with varying concentrations of vancomycin and for *L. plantarum* with varying concentrations of D-alanine and a constant concentration of 256mg/L vancomycin.

Results:

The *L. casei* was initially supplemented with 256mg/L vancomycin to test the natural resistance of the bacterium. After allowing growth overnight, the optical density was an average of 1.992. It was also supplemented overnight with solely D-alanine at concentrations ranging from 100mM to 5mM, all in 1mL of MRS broth. As was determined with the pure vancomycin, the optical densities were greater than 1.500 for all the pure D-alanine solutions.

An assay was performed with vancomycin in the absence of D-alanine to determine the concentration of antibiotics needed to overcome the resistance. The vancomycin was scanned from 0mg/L to 1024mg/L. The resulting absorbance

values are shown in Figure 1. In general, the absorbance values decreased as the concentration approached 1024mg/L.

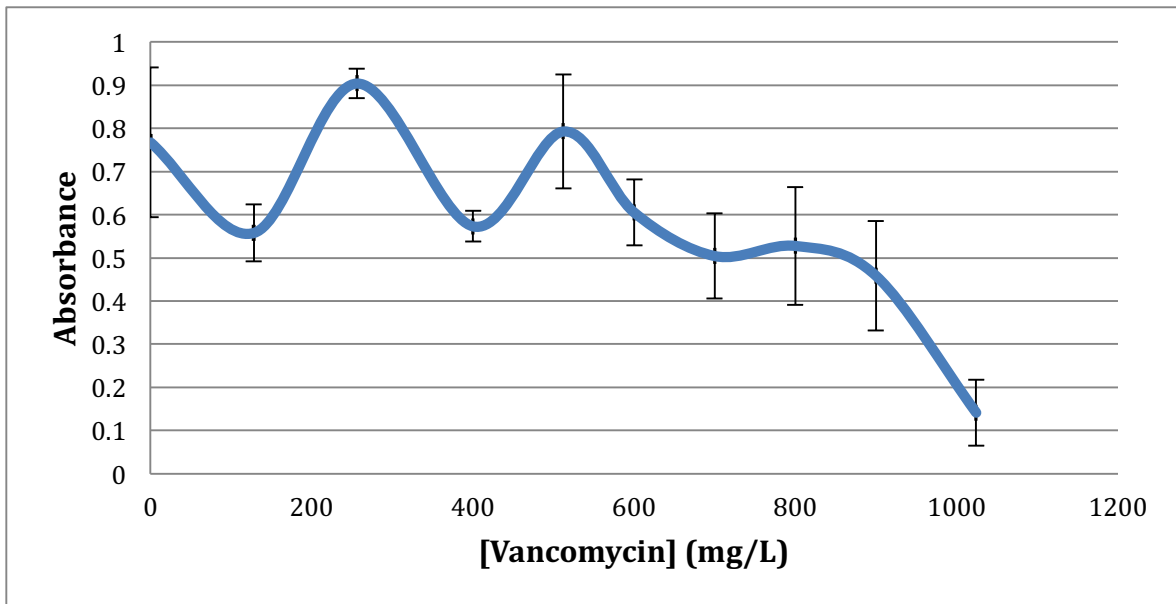


Figure 1: The killing curve of *L. casei* supplemented with vancomycin in the absence of D-alanine is shown here.

For non-resistant bacteria, the minimum concentration required for killing is theoretically a great deal lower than 1000mg/L. To demonstrate how non-resistant bacteria function in the presence of vancomycin, an assay was performed with *B. subtilis*. It was incubated with vancomycin only, at concentrations ranging from 0mM to 16mM. The resulting absorbance values are graphed in Figure 2. The absorbance values dropped to approximately zero around 0.5mg/L vancomycin.

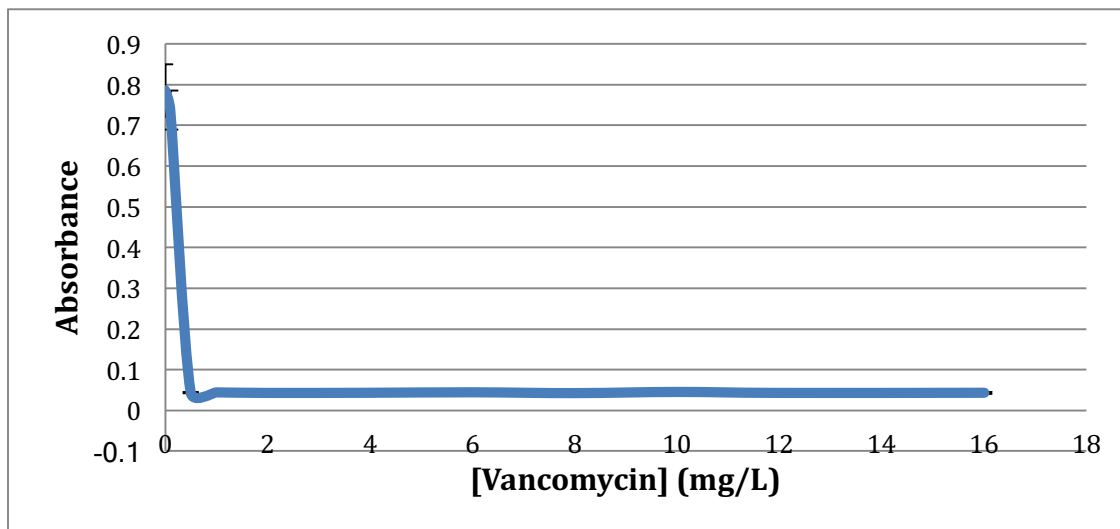


Figure 2: The killing curve of *B. subtilis* supplemented with vancomycin ranging from 0mM to 16mM is shown here.

Next, to determine if supplementation with the amino acid D-alanine results in outcompeting of the D-lactate in the synthesis of the dipeptide, an assay was performed with the concentration of the vancomycin held constant at 256mg/L. At the same time, the concentration of the D-alanine was altered from 0mM to 20mM. After the overnight incubation, there was no growth observed for any concentration of D-alanine. The only growth was seen with the exclusively vancomycin sample. The results are graphed in Figure 3. The absorbance decreased to approximately zero between 1mM and 2mM D-alanine.

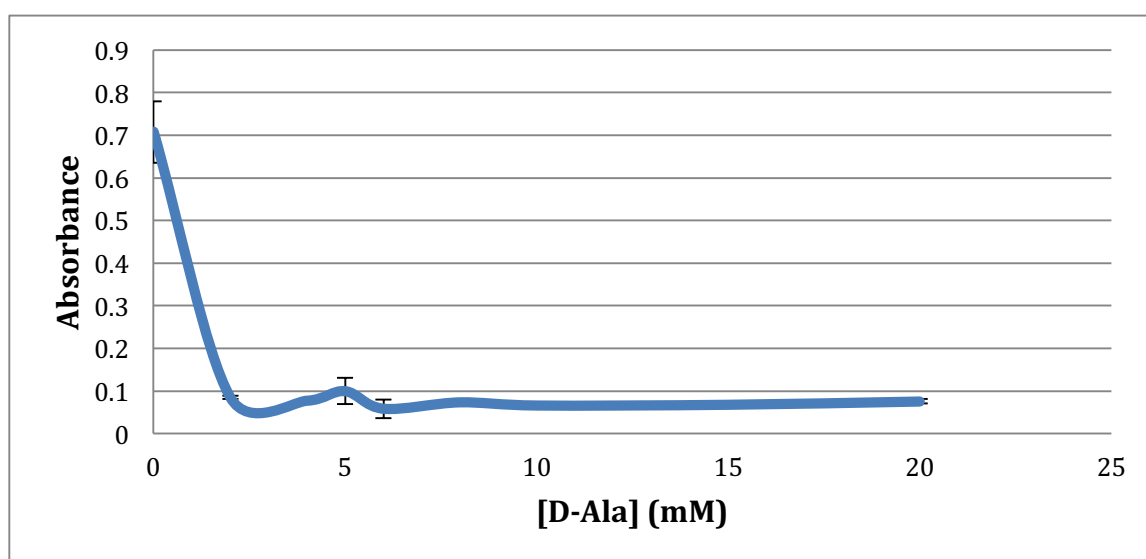


Figure 3: The killing curve of *L. casei* supplemented with 256mg/L vancomycin and varying concentrations of D-alanine is shown here.

To test if lower concentrations of vancomycin would still produce the same results, an assay was performed with a constant D-alanine concentration of 5mM. Vancomycin was varied, using concentrations of 128mg/L, 64mg/L, 32mg/L, 16mg/L, and 1mg/L. After the overnight incubation, the optical density values were recorded. The results are shown in Figure 4. There was less growth observed as the concentration of the vancomycin increased, with negligible amounts of growth observed above 128mg/L vancomycin.

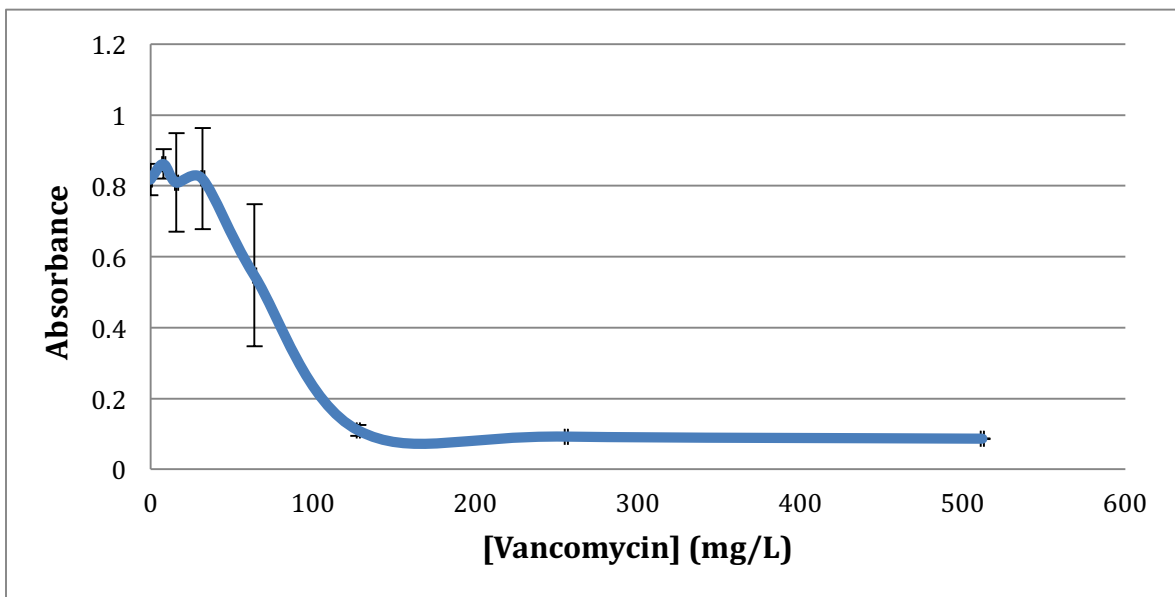


Figure 4: The killing curve of 5mM D-alanine with the alteration of the vancomycin concentration is shown here.

The concentration of D-alanine was then raised in an attempt to lower the vancomycin concentration. Vancomycin concentrations of 32mg/L and 16mg/L were tested, with scanning from 0mM D-alanine to 50mM D-alanine, and 8mg/L was tested with scanning from 0mM to 30mM. Figure 5 illustrates the absorbance values obtained from the three samples after overnight incubation. Both the 16mg/L and 32mg/L samples had absorbance values that dropped off to approximately zero,

while the 8mg/L absorbance remains relatively the same with varying D-alanine concentrations.

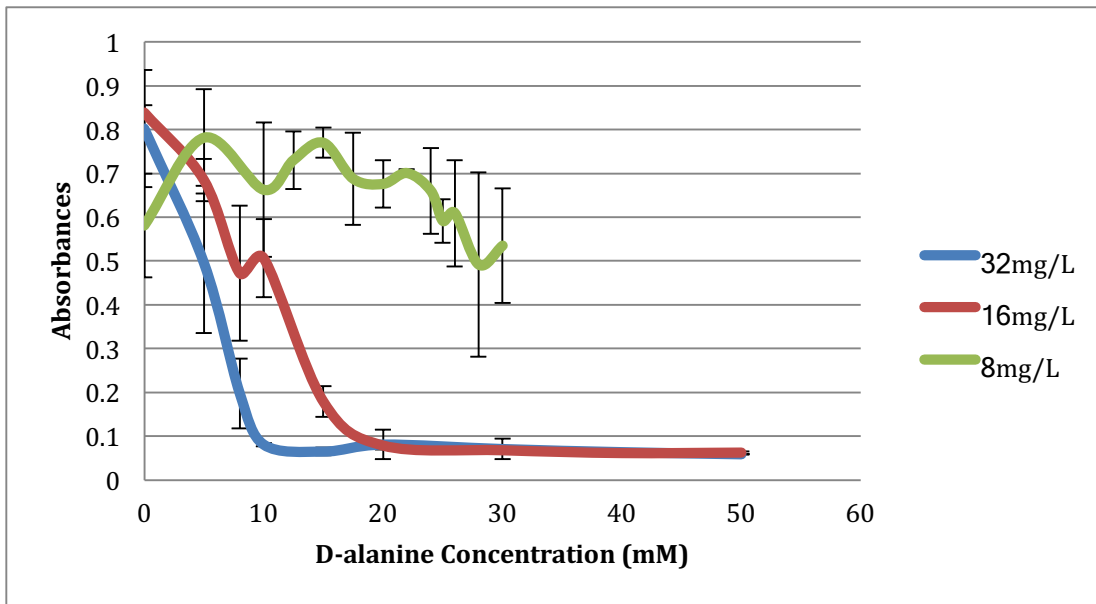


Figure 5: The killing curves of 32mg/L, 16mg/L, and 8mg/L of vancomycin with varying D-alanine concentrations are shown here.

With *L. plantarum*, the concentration of D-alanine was varied from 0mM to 5mM, with the focus of the points between 0mM and 1mM. The optical density results are graphed in Figure 6. There was no change in the absorbance values, even as the concentration of D-Alanine increased.

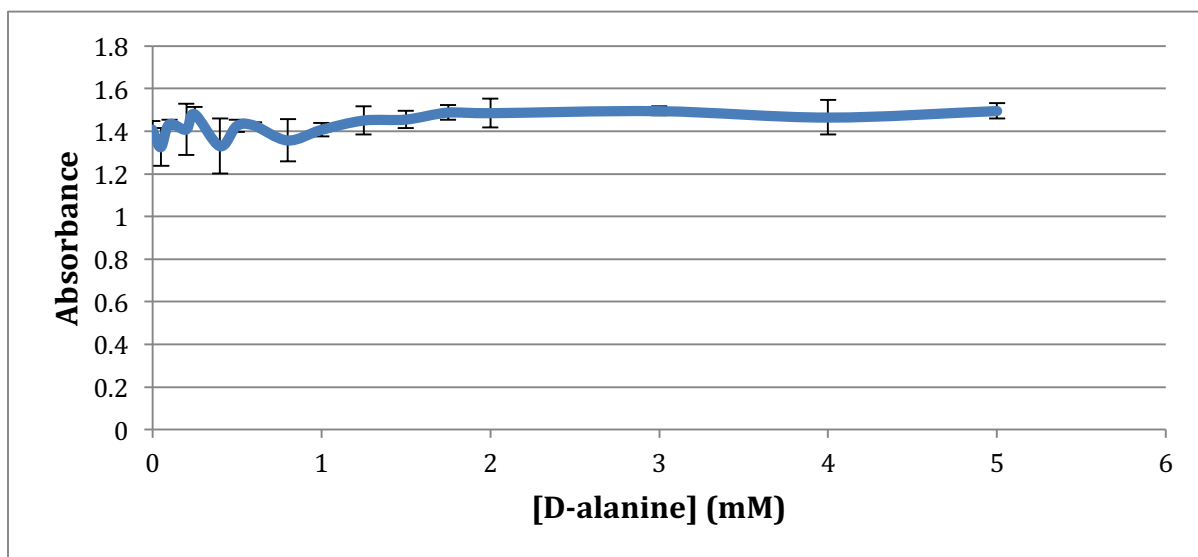


Figure 6: The *L. plantarum* killing curve at a vancomycin concentration of 256mg/L with varying D-alanine concentrations is shown.

In an attempt to further optimize and improve upon this method, the production of D-Ala-D-Lac in the bacteria was supplemented with the dipeptide D-Ala-D-Ala. For this, the bacteria were supplemented with 256mg/L vancomycin and a range of D-Ala-D-Ala concentrations. The D-Ala-D-Ala concentrations used were 5mM increments between 0mM and 20mM and 10mM increments from 20mM to 50mM. After incubating overnight in a shaker, all the wells exhibited a high degree of growth. All the absorbance values fell above 1.500.

Discussion:

From the vancomycin and D-alanine single agent toxicity assays, it can be determined that neither will impact the growth of the bacteria *L. casei* alone. The high absorbance values are indicative of high growth levels, and therefore no killing. These experiments were performed to confirm that the bacterium was capable of growing in the presence of each compound individually. As a result, when added to the bacteria together, any observed killing could be attributed to the combination of the two components.

To reinforce these findings and to determine the minimum concentration of vancomycin needed to overcome resistance without any alterations, an assay was performed with varying vancomycin concentrations in the absence of D-alanine. The bacteria continued to grow at relatively high rates in the presence of vancomycin until the concentration was 1024mg/L. As a result, the *L. casei* bacterium is resistant to vancomycin up to approximately 1000mg/L. At that high concentration, the bacteria will be killed off, but, in the human body, this concentration is not feasible. The vancomycin must be at concentrations below 20mg/L to be functional in the

human body, as the typical dosage of the antibiotic used to treat bacterial infections in adults is 15-20mg/L.¹⁵

B. subtilis is a bacterium that is not resistant to vancomycin. It has a high sensitivity to the antibiotic, as the pentapeptide chain has the target D-Ala-D-Ala C-terminus. By 0.5mg/L vancomycin, the bacteria exhibited no growth. Therefore, the minimum concentration of vancomycin required to kill the bacterium is much lower than that required for *L. casei*. This data is used to verify the resistance of the *L. casei* and to serve as a model for the typical concentrations when vancomycin is functioning normally to inhibit growth of a bacterium.

With 256mg/L vancomycin and D-alanine supplementation, killing was observed by 2mM D-alanine. The vancomycin concentration is greatly lowered from the value of 1000mg/L determined from the vancomycin single agent toxicity assay. As a result, the D-alanine does affect the activity of the vancomycin. It increases the function of the antibiotic, indicating that D-alanine is capable of outcompeting the D-lactate in the biosynthetic pathway of the bacteria in order to be incorporated into the peptidoglycan layer. The peptidoglycan layer is incorporating the dipeptide D-Ala-D-Ala at the C-terminus, rather than D-Ala-D-Lac. This demonstrates the initial aim of the research: D-alanine can be supplemented with vancomycin to increase antibiotic activity. The concentrations are still beyond what is achievable in the human body though. The next step was to determine if there is a combination of vancomycin and D-alanine that kills the bacteria and that is non-toxic to the human body.

To establish how low the vancomycin concentration could be while still having an effect, an analysis was performed at 5mM D-alanine. This is a potential concentration for surface infections and showed significant levels of killing at 128mg/L. Below that concentration, a substantial amount of growth was observed. Though 5mM D-alanine is feasible, the concentration of vancomycin is still too high to be used in the body.

D-alanine is possible on topical infections at concentrations as high as 20mM. Therefore, if the concentration of D-alanine is raised from 5mM, the vancomycin may be able to be lowered to values that are functional for human bodies. To see if lower vancomycin concentrations still resulted in biologically possible D-alanine concentrations, 32mg/L, 16mg/L, and 8mg/L vancomycin were tested. It was determined that the 32mg/L vancomycin killed the bacteria at concentrations of approximately 10mM D-alanine and higher. For 16mg/L, the vancomycin killed the bacteria at D-alanine concentrations of approximately 18mM and higher. From this data, it was determined that 16mg/L vancomycin and 18mM D-alanine will inhibit the growth of *L. casei*. The vancomycin concentration is within the typical dosage range, but the D-alanine concentration is too high for use in the blood. However, it is potentially usable on skin-level infections. For real world applications, lower concentrations would be preferable. The 8mg/L assay was inconclusive. Higher concentrations of D-alanine would have to be tested to determine the point where absorbance decreases to approximately zero. From the data obtained, it appears that the concentration that inhibits the growth of the bacteria will be greater than a biologically usable value.

L. plantarum, with the D-Ala-D-Ala-dipeptidase, is a more accurate model of the active mechanisms that occur in vancomycin-resistant bacteria. It did not show any killing with 256mg/L vancomycin, even as the concentration of the D-alanine increased to 5mM. *L. casei* had crosslinking of the peptidoglycan layer inhibited at concentrations of 2mM and higher. The goal with the *L. plantarum* was to determine if the D-alanine was capable of replacing the D-lactate at the surface. For *L. casei*, the D-alanine was outcompeting the D-lactate on an intracellular level. The bacteria were utilizing the D-alanine to form a precursor that was then attached to the peptidoglycan layer. By supplementing with D-alanine, enough of the desired dipeptide was being formed that VanXYc was unable to hydrolyze all the bonds. *L. plantarum* is unable to overcome resistance in the same manner. The D-Ala-D-Ala-dipeptidase, in combination with VanXYc, degrades any D-Ala-D-Ala dipeptide that does form before it can be incorporated into the peptidoglycan layer. As a result, for vancomycin to be effective, the transpeptidase would have to cleave the D-Ala-D-Lac peptide bond at the surface and then incorporate the supplemented D-alanine. As viability assays performed with similar concentrations did not exhibit killing, the transpeptidase did not successfully replace the fifth position amino acid.

To further optimize and improve upon this method, the production of D-Ala-D-Lac in the bacteria was supplemented with the dipeptide D-Ala-D-Ala. The goal was to eliminate the need to outcompete the D-lactate by directly providing the necessary dipeptide. Therefore, the competition step occurs at the incorporation into the pentapeptide precursor. If the bacteria could be made to incorporate the desired dipeptide over the D-Ala-D-Lac dipeptide, lower concentrations than those

needed for the single amino acid may be sufficient. Lower concentrations allow for this method of combating antibiotic resistance to be more biologically feasible and more efficient for use in the human body. From the recorded data, the dipeptide failed to outcompete the D-Ala-D-Lac and as a result failed to facilitate killing in conjunction with vancomycin. The bacteria showed growth, even at high concentrations of the dipeptide and the vancomycin. With the amino acid D-alanine, there is competition over the formation of the dipeptide precursor. The D-lactate and the D-alanine present in the system both attempt to form peptide bonds with additional D-alanine. It has been shown that the single amino acid is able to outcompete the D-lactate in the formation of this bond. With the bacteria continuing to grow in the presence of the dipeptide and vancomycin, it can be determined that the formation of the pentapeptide layer is favorable for the D-Ala-D-Lac, despite the presence of excess D-Ala-D-Ala. As an additional consideration, the D-Ala-D-Ala is larger than the single amino acid, and therefore has a lower permeability into the bacterial cells.

Conclusion:

L. casei is a vancomycin-resistant bacterium up to the high concentration of 1000mg/L. When compared to a non-resistant bacterium, the vancomycin concentration is a great deal higher. To lower the amount needed, supplementation with D-alanine functions to outcompete the D-lactate amino acid in the formation of the peptide bond prior to the attachment of the dipeptide to the pentapeptide chain. As a result, supplementation of vancomycin treatments with D-alanine has the potential to overcome resistance. The lowest combined concentrations shown to

inhibit growth of the bacteria are 16mg/L vancomycin with 18mM D-alanine. These concentrations are higher than desired, but are still usable for superficial infections. For use in the bloodstream, lower D-alanine concentrations are required.

In an attempt to lower the concentration needed, the D-Ala-D-Ala dipeptide was used. It failed to inhibit the growth in conjunction with vancomycin, indicating that the attachment of the precursor dipeptide to the pentapeptide layer is favorable for the D-Ala-D-Lac dipeptide when both are present. With *L. plantarum*, a different mechanism of replacing the D-lactate was tested. Growth was not inhibited, indicating the optimal mechanism to replace the D-lactate amino acid at the C-terminus of the peptidoglycan layer is in the formation of the precursor prior to incorporation into the cell wall.

As this method has been shown to work for concentrations feasible on topical infections, the next step is to test it on bacteria that have acquired vancomycin resistance. When these bacteria are supplemented with D-alanine and vancomycin, will they act more like *L. casei* or *L. plantarum*? Overall, overcoming vancomycin resistance was demonstrated to be feasible in intrinsically resistant bacteria that do not degrade the precursor D-Ala-D-Ala dipeptide, though additional research must be performed to determine the optimal manner to achieve biologically usable concentrations and to determine how bacteria that have acquired resistance will behave.

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